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The Effects of High Cheese Diets on Brain Serotonin and Alcohol Consumption

Beverly A. Utt

Abstract

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Male weanling rats were placed on a nutritionally adequate casein diet and randomly assigned to one of eight groups. The first four groups were force fed alcohol for 22 days prior to the experimental phase of the study, while the last four groups had access to water only. Upon commencing the experimental phase each group was given one of the following diets: continuation of the casein diet; Cheddar; Limburger; or Roquefort cheese diets, along with the choice of alcohol and/or water. The particular cheeses were chosen for their ability to raise brain serotonin levels (62). Alcohol consumption was measured daily among all groups.

A general linear hypothesis was used to compare serotonin levels with diet, drinking status, animal, and alcohol consumption per day. Of the variables considered in the analysis, the most important variable in terms of accounting for variability in serotonin levels was diet, i.e. whether the animal was given access to a casein or cheese diet, however, it failed to reach significance ($p=.08$). Findings from this study suggest that serotonin levels are the result of an

interaction between type of diet and total alcohol consumption, i.e., whether the animal drank alcohol throughout the entire course of the experiment, or merely had access to it during the experimental phase.

LOMA LINDA UNIVERSITY

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THE EFFECTS OF HIGH CHEESE DIETS ON BRAIN SEROTONIN AND ALCOHOL CONSUMPTION

by

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Each person whose signature appears below certifies that this thesis in his/her opinion is adequate, in scope and quality, as a thesis for the degree Master of Science.

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I. REVIEW OF LITERATURE

Introduction

The brain is extremely responsive to environmental inputs. Researchers are suggesting that, even after the consumption of a meal, predictable changes in brain composition occur depending on the foods consumed. If it is true that consumption of a meal produces changes in blood composition, which in turn produce changes in brain composition, then it would by reasonable to assume that the changes in brain composition may result in changes in functional activity of the brain (1).

Until recently, this sort of thinking would have been considered radical. Previously it was thought that the brain extracted needed nutrients from the bloodstream in an unstructured fashion, i.e., without considering the concentration of those nutrients in the blood. So for this reason the changing concentrations of various nutrients in the blood were not considered to directly influence the brain (2).

Nutritional research has been more concerned with the long-term effects of diet on the brain. Studies have produced correlations between inadequate diets and retarded development of the brain, which, if severe enough, result in behavioral and learning deficits.

Serotonin and its precursor tryptophan, have been identified as constituents that are altered depending on the foods

consumed (3). This response occurs rapidly because of the following mechanism. The first step in the synthesis of serotonin appears to be the rate-limiting step. The enzyme catalyzing this step is relatively unsaturated with substrate, so that fluctuations in brain tryptophan levels rapidly affect whether or not serotonin is synthesized (1).

These observations have led to an interesting theory. Previously it was thought that behavior was controlled by brain biochemistry. However, some researchers are suggesting that behavior can control brain composition, and may thus serve to maintain homeostasis within the individual (3).

Purpose of the Study

It is the purpose of this study to observe the effects of diets containing large amounts of cheese (utilizing three different cheeses known to have high tyramine and other amines) on brain serotonin levels in rats. Studies by Myers' group (4) and Fray, et al. (5), have suggested that the serotonergic systems in the brain may be involved in the preference and/or consumption of alcohol. On the other hand, Pickett, et al. (6), have suggested that differential alcohol influences on serotonin metabolism or turnover rate may result in differing preference for alcohol. In light of these studies, it is also the purpose of this study to examine the response of

ethanol consumption to note any relationships between altered serotonin metabolism and subsequent alcohol-seeking behavior.

Cheese - Definition and Classification

Cheese represents a large part of the American diet. Figures for 1975 from the Economic Research Service indicate that Americans were consuming approximately 16 pounds per year (7).

> Cheese may be defined as the concentration of all or part of the components of milk obtained through the coagulation of the major milk protein, casein, by suitable enzymes and/or by acid produced by bacteria. The curd, separated from the whey, is used at once in unripened cheese. In other cheese, the curd is ripened by the action of beneficial bacteria, molds, yeasts and enzymes (8).

Because of the wide diversity in characteristics, more than 2000 names have been given to cheeses. And these have been classified into 10 to 18 distinct types depending on the methods of manufacturing (9).

Other variables are the basis for classification systems also. These variables include composition (determined by moisture, fat and calcium), age, texture or general appearance, type of milk used, type of ripening agent used, and country of origin (9).

Composition and Nutritive Value of Cheese

In the process of cheese-making many of the nutrients found in the original milk source are retained, some concentrated in the final cheese product, and some are lost. Most cheese retains a large part of the milk protein, the minerals, fat-soluble vitamins, and significant amounts of water-soluble vitamins. When the curd is separated from the whey many of the nutrients become concentrated in the curd. This results in an eight-to-ten-fold increase in protein, fat, calcium, phosphorus, and vitamin A over those in the original milk source. Those nutrients that are, to some extent, lost during cheese-making are lactose, the soluble proteins, and water-soluble salts (8).

Cheese is considered to be a high quality protein and represents the most concentrated form of nitrogenous food. The protein content of commonly used cheese ranges from 2.10 to 8.14 grams per ounce, while 5.00 to 7.00 grams seems to be the protein content range for most cheeses (8). The type of protein found in cheese is mainly casein.

The fat content of cheese appears to depend on the original fat content of the milk from which the cheese was made (8). The fat-soluble vitamins are retained due to the fact that most of the fat of the original milk source is retained. Because of this, cheese is a good source of vitamin A.

Water-soluble vitamin content of milk is altered in cheese-making when the curd is removed from the whey. Each type of cheese varies in its content of water-soluble vitamins depending on the following factors: the microorganisms used as starter cultures and as ripening agents; and the amount of ripening (10).

Losses have been reported to occur in thiamine, riboflavin, niacin, biotin, vitamin B_6, pantothenic acid and folic acid (8). Evans, et al., found that only 8.8% of the original thiamine remained, and that 43 to 73% of that thiamine was lost over the next 12 months (11).

Characteristics of Cheddar, Roquefort and Limburger

Cheddar cheese is a hard cheese, usually yellow in color, but can range from white to yellow. Its milk source is sweet, whole cow's milk, either raw or pasteurized. Research has shown that the pasteurized milk produces a better quality cheese. In the United States, most of the Cheddar cheese has been made from heat-treated or pasteurized milk (12). An analysis of Cheddar cheese reveals the following: moisture, 37 to 38 percent (not more than 39 percent); fat, 32 percent (fat in the solids not less than 50 percent); protein, 25 percent; and salt, 1.4 to 1.8 percent (7,12).

Limburger is a semisoft cheese. Limburger's unmistakable characteristics include a strong aroma and flavor. Limburger is surface ripened, meaning that ripening starts at the surface, and progresses to the center. This is accomplished by first spraying the surface of the cheese with a culture of yeasts, followed by a culture of Bacterium linens. Analysis yields the following: moisture, not more than 50 percent (usually 43 to 48 percent); fat, 26.5 to 29.5 percent (not less than 50 percent of the solids); protein, 20 to 24 percent: ash, 4.8 percent; and salt, 1.6 to 3.2 percent (12).

Tyramine in Cheese

The presence of tyramine in cheese is due to the decarboxylation of tyrosine. It is thought, by some, that some of the bacteria which break down the casein, have amino acid-decarboxylase activity. As a result, amines are produced, and we get tyramine from tyrosine (13,15).

According To Lagerburg, et al. (14), lactic acid bacteria produce no tyrosine decarboxylase. However, the coliform organisms and Group D streptococci are responsible for producing this tyrosine decarboxylase.

The concentration of tyramine in cheese is related to three factors: the ripening or maturation time; bacterial flora; and manufacturing process (13).

There is a popular misconception that cheese with obvious visible and olfactory evidence of putrefaction contains the most tyramine. This is not necessariy true according to Blackwell and Mabbitt (15), nor is flavor an accurate indicator of tyramine content.

The tyramine content of cheese varies from 0 ug per gram in cottage cheese to about 2000 ug per gram for Camemburt cheese (16). The approximate level of tyramine found in New York Cheddar cheese is 1416 ug per gram cheese (15).

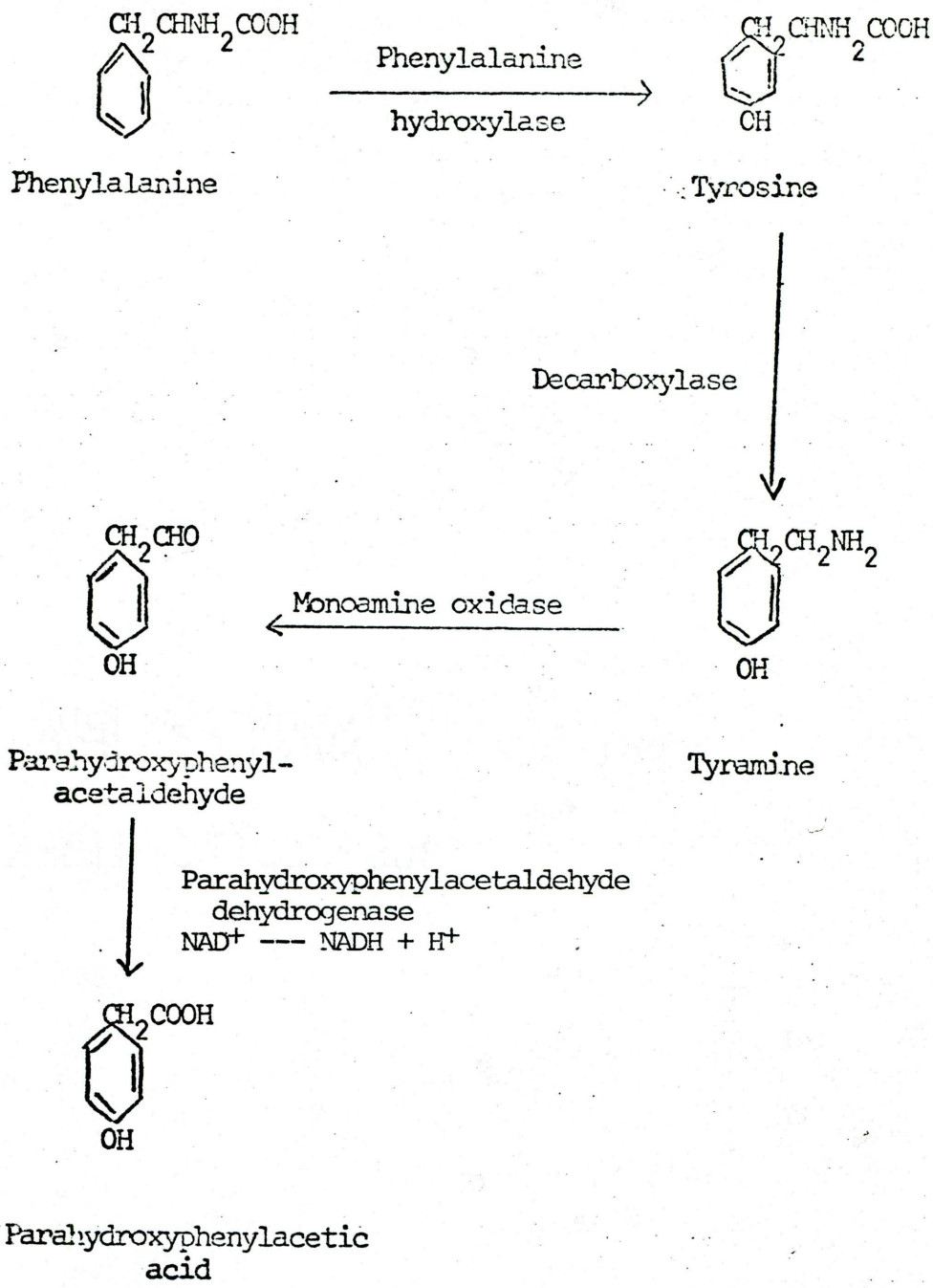

Figure 1. Metabolism of Phenylalanine
(Adapted from Stryer 17)

Effects of Tyramine

Tyramine acts as a powerful vasopressor. In the human body, tyramine is metabolized with the aid of monoamine oxidases. If these enzymes are inhibited by any of the drugs used to treat patients with various types of depression, then the tyramine from cheese or other food sources is free to exert its hypertensive effects on the system. Besides causing hypertension, tyramine may be responsible for causing headaches, palpitation, nausea and vomiting in certain people (15). It has been suggested that the pressor effect exerted by the MAO inhibitors which impede the normal degradation of tyramine to non-toxic parahydroxyphenylacetic acid, may be enhanced one hundred fold (18).

It is also interesting to note that tyramine can be absorbed from the mouth. In a study done by Price and Smith (19) it was noted that about 5% of the tyramine content of cheese may be absorbed from the mouth, which means it would bypass the monoamine oxidase in the intestines and liver. This suggests that if the tyramine content of the cheese being consumed was high, enough tyramine could be absorbed to exert a systemic effect.

Serotonin - Synthesis and Metabolism

Serotonin has been found in the enterochromaffin cells of the gut, pineal parenchymal cells, platelets, and certain brain neurons (20). Serotonin has the ability to act as a powerful vasoconstrictor as well as stimulating smooth muscle contraction (21). Within brain neurons, serotonin functions as a neurotransmitter. Neurotransmitters transmit signals across synapses to other neurons within the brain or to muscle cells or secretory cells outside the brain (20).

Serotonin is unable to cross the blood brain barrier. Therefore, serotonin must depend on the transport of its precursors across this barrier for the production of serotonin in the brain to occur (21). To test if this were true or not, relatively large amounts of peripherally administered serotonin (60 mg/K) were given to a group of rabbits and dogs. No observable increase in brain serotonin occurred (22).

Fernstrom and Wurtman (2) have suggested that most of the serotonin present in the brain is confined to a distinct group of neurons known as raphe nuclei. The cell bodies of the raphe-nuclei neurons are located in the brain stem.

L-tryptophan is the essential amino acid from which serotonin is synthesized. The steps of serotonin synthesis include the following (See figure 2). The first step, which serves as the rate-limiting step, involves the hydroxylation

of tryptophan to 5-hydroxytryptophan; this compound is decarboxylated to form serotonin (5-HT). The hydroxylation reaction is catalyzed by the enzyme tryptophan hydroxylase, which is found only in serotonin-containing cells; and the decarboxylation reaction is catalyzed by a widely-distributed decarboxylase enzyme. Further metabolism involves the deamination of 5-HT by monoamine oxidase (MAO), producing an aldehyde intermediate, and finally oxidation to 5-HIAA, which is catalyzed by aldehyde dehydrogenase (20).

Availability of the 5-HT Precursor Tryptophan

The synthesis of serotonin is very much dependent on the availability of its precursor tryptophan. The existing interneuronal concentrations of tryptophan may not be sufficient to saturate the enzyme, because the concentrations of tryptophan normally present in the brain are below the Michaelis constant (Km) of tryptophan hydroxylase. Thus, even small variations in the availability of tryptophan appear to rapidly affect the rate of 5-HT synthesis (23,24,25,26).

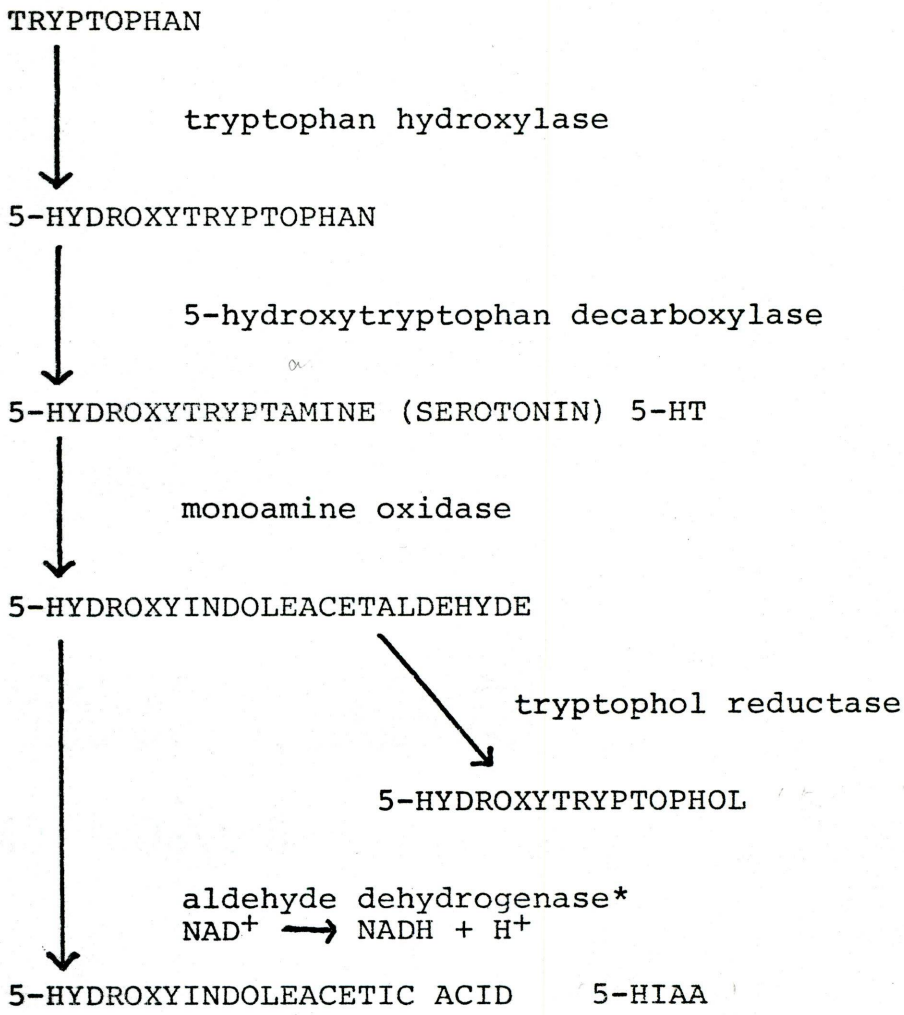

Figure 2. Oxidative and reductive pathways of serotonin metabolism (Adapted from White, 29)

*Oxidative deamination blocked here by the presence of the ethanol metabolite, acetaldehyde.

Control of Serotonin Biosynthesis in Brain

The first step in the synthesis of 5-HT is important in that it appears to act as the rate-limiting step. The rate of this reaction is largely dependent on the availability of its substrate, L-tryptophan. This is to say, that changes occurring in the synthesis of 5-HT are related to the available tryptophan (20,24,25,28).

Effects of the Diet on Brain Serotonin

The brain is extremely responsive to environmental inputs. Researchers are suggesting that, even after the consumption of a meal, predictable changes in brain composition occur depending on the foods consumed. We know that serotonin is one of the constituents that is affected (1).

The effect of diet on the brain may be related to the effect the diet has on the availability of 5-HT's precursor, tryptophan.

The effects of administering insulin on the plasma constituents are well known - the concentration of glucose is lowered and there is a stimulation of most amino acids into muscle, resulting in a fall in plasma concentration of these amino acids (20). Tryptophan, on the other hand, exhibits a different response. When rats were injected with insulin or given access to a carbohydrate diet, their plasma tryptophan

increased (2). Using the same treatment, researchers are now able to show subsequent increases in the concentration of brain tryptophan and serotonin (30).

However, plasma tryptophan is not the only factor responsible for the brain concentrations of tryptophan and serotonin. This was borne out by the following line of reasoning. If plasma tryptophan alone determines brain tryptophan and serotonin, the addition of protein to the diet should raise tryptophan by two methods: First, by causing insulin to be secreted, and secondly by contributing tryptophan molecules (3). To observe the effect protein had on subsequent brain tryptophan and 5-HT, rats were placed on a diet containing carbohydrate and protein in the form of casein. The results showed that plasma tryptophan rose, higher than those occurring after consumption of a carbohydrate meal, but there were no subsequent increases in brain tryptophan or 5-HT (20). These results led to the following explanation. The effect of diet on brain tryptophan and 5-HT is best determined by the ratio of tryptophan in the diet to the sum of neutral amino acids that compete with tryptophan for transport into the brain. In the case of a protein-containing diet, the diet would most likely be contributing more neutral amino acids compared with tryptophan. This would increase the denominator of this fraction more than the numerator, thus allowing entry of more neutral amino acids into the brain than tryptophan (3).

In regards to dietary fat, it appears that none of Fernstom's dietary alterations (20) elicited a rise in brain tryptophan, even though serum nonesterified fatty acid levels and serum free tryptophan concentrations increased in proportion to the fat content of the diet.

Functional Activity of Brain 5-HT

Serotonin functions in a variety of ways in the human body. Not all of its functions are clearly understood. However, it appears that an excess may stimulate cerebral activity and a deficiency has a depressant effect (21).

This has been demonstrated in rats after treatment with tryptophan and an MAO inhibitor, tranylcypromine. Upon treatment, brain 5-HT rose, accompanied by hyperactivity behavior measure on activity meters (24,31,32). The appearance of this hyperactivity was dependent on all of the following: MAO inhibition; tryptophan administration; tryptophan hydroxylase activity; and 5-hydroxytryptophan decarboxylase activity (27,31,32). Other investigators have observed similar results (33,34,35).

An explanation for this hyperactivity is as follows. Normally an excess of tryptophan can be handled by intraneuronal binding and oxidative deamination. When MAO is inhibited, 5-HT accumulates at post-synaptic receptors, and hyperactivity occurs as a result (24).

In contrast to the hyperactivity syndrome, Brodie (36) suggests another biologic function of serotonin. Brodie bases his interpretation on the concepts of Hess (36). Hess postulated that reactions of the organism to environmental changes are controlled by a subcortical system. The subcortical system consists of two mutually antagonistic divisions - the ergogrophic and trophotropic. The ergotrophic division produces arousal, increased sympathetic activity, enhanced skeletal muscle tone and activity, and an over-all state of excitement. Whereas the trophotropic division produces behavioral patterns just the reverse of the above. The over-all effects of trophotropic stimulation are sedation, increased parasympathetic activity, and decreased muscle tone and activity. Serotonin supposedly acts as the regulator of this system.

In an attempt to explain these opposing views, Grahame-Smith (31,32) has suggested the following. From Brodie and Reid's (36) study, they base some of their argument upon the correlation they found between the lowering of brain 5-HT concentration and the development of the syndrome of reserpine sedation, assuming that this is caused by the release of 5-HT onto its sites of activity. However, knowing that 5-HT can produce an excitatory state and assuming that MAO can metabolize functionally inactive 5-HT, Grahame-Smith came up with the following explanation. If reserpine released 5-HT from its binding site not onto its site of activity but onto MAO by which it was metabolized without becoming functionally

active, then the amount of 5-HT available would be lowered and sedation would occur (31,32).

Effect of Serotonin on Ethanol Consumption

Some researchers have suggested that 5-HT may play a role in an animal's selection of ethanol (4). Their argument is supported by observations that the concentrations of 5-HT and 5-HIAA in the brain are higher in ethanol-selecting strains of rats and mice than in water-selecting animals. Some have gone a step further and suggested that ethanol drinking increases brain 5-HT concentration in ethanol-preferring rats and mice, but does not affect the concentration in the brains of water-selecting animals (37). Attempts to relate 5-HT to alcohol consumption have, however, produced controversial findings; reports of the most prominant work will be reviewed here.

Myers and Veale (4) report that when rats were given p-chlorophenylalanine (pCPA 300 mg per kg), the preference for ethanol was significantly reduced in some and abolished in others. pCPA depletes brain serotonin by inhibiting the enzyme tryptophan hydroxylase. These results were confirmed by the work of Frey, et al. (5). The reasons for the long term action of pCPA in reducing preference for alcohol are not known. Myers and Veale (4) have offerred the following

explanation. They suggest that pCPA may cause 5-HT to be depleted in one or more of the limbic structures involved in drinking. If alcohol also reduces brain 5-HT, as some have suggested, then perhaps the rats treated with pCPA rejected alcohol because its intake would have further lowered already-depleted levels of serotonin. The animals may have been atempting to conserve the remaining stores of serotonin (4).

Other studies have yielded conflicting results. The results obtained on alcohol preference are not consistent with the effects of pCPA. Nachman, et al. (38), and Geller (39) were unable to confirm Myers' and Veale's (4) findings where pCPA significantly reduced preference for ethanol. Nachman suggested that a conditioned aversion might be established through the pairing of ethanol or saccharin with the administration of pCPA or other noxious substances (39). Results of Parker and Radow (40) also support Geller's contention that the reported effects of pCPA on the rat's preference for ethanol may have been due largely to the animals acquiring conditioned aversions to ethanol during pCPA treatments.

The effects of an intracisternal injection of 5,6-dihyroxytryptamine (5,6-DHT; 75 ug per rat) on alcohol preference were studied in rats by Ho, et al. (65). Results indicated that alcohol consumption was increased significantly from about the 5th to 11th days after treatment. Since both pCPA and

5,6-DHT produce depletion of brain 5-HT, Ho's findings on the increase in alcohol selection appear to be in agreement with the observation by Geller (39) noted above.

It has been suggested by Hill, et al. (41), that pCPA may present limitations in testing the independent effects of serotonin depletion on ethanol consumption. The results of a study by Hill, et al. (41), offered no support for the hypothesis that pCPA induces avoidance of alcohol. In fact, analysis of the data by ethanol concentration indicated a significant increase in consumption at the most highly selected concentration i.e., 5% (41).

In a study relating the effects of tryptophan on the selection of ethyl alcohol in different strains of rats (42), the conclusion drawn was that tryptophan may exert its effect on ethanol intake through an alteration in the metabolism of cerebral serotonin, but such an effect seemed to be dependent upon the strain of rat tested.

Finally, Pickett, et al. (6), and Kiianmaa (37) have provided evidence against the hypothesis that brain serotonin content influences alcohol preference. However, they do not preclude the possibility that differential alcohol influences on serotonin metabolism or turnover rate may result in differing preference for alcohol.

Metabolism of Ethanol

Alcohol is oxidized primarily in the liver (80-90%). Metabolism of ethanol involves two steps: an initial oxidation to acetaldehyde by means of alcohol dehydrogenase (ADH), followed by oxidation to acetate, with the aid of aldehyde dehydrogenase (See Figure 3). Both the ADH and the aldehyde dehydrogenase reactions are dependent upon the availability of NAD^+ as hydrogen acceptor. Oxidation of ethanol will thus alter the equilibrium between NAD^+:NADH in the cytoplasm (43).

The rate-limiting step for alcohol oxidation does not appear to be the activity of the ADH enzyme alone, but rather the reoxidation of the ADH-NADH complex. The hydrogen that is formed from the reoxidation of NADH to NAD^+ must be transported to the mitochondria where it enters the respiratory chain (43).

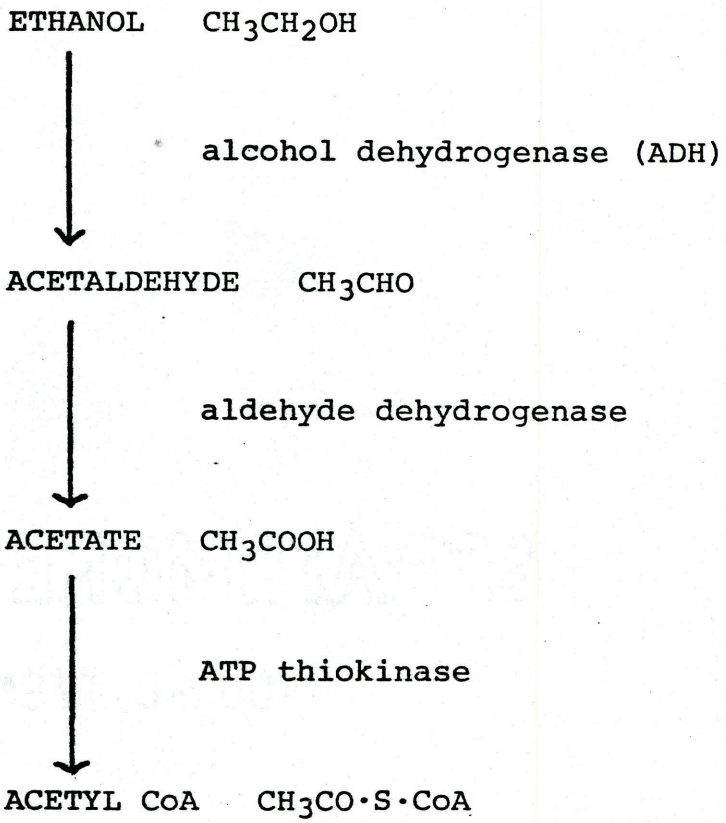

Figure 3. Metabolism of Ethanol (Adapted from Lahti 43)

Serotonin Catabolism and the Effects of Ethanol and Acetaldehyde

The biogenic amines, norepinephrine, dopamine and serotonin, all follow very similar breakdown pathways. Through the action of monoamine oxidase, these amines are oxidatively deaminated to their corresponding aldehydes. The next step depends on the enzyme involved. If the aldehyde is acted on by aldehyde dehydrogenase, a carboxylic acid will be formed. If, however, the aldehyde is reduced by an alcohol reductase, then an alcohol will be formed. Examination of urinary excretion indicates that the catabolic pathway yielding an acid is the major pathway (43).

Many studies on the effect of ethanol on the catabolism of the biogenic amines were initiated as a consequence of the finding of Olson, et al. (44), that chronic alcoholics excreted significantly less 5-HIAA, the acidic metabolite of serotonin, in their urine than did non-alcoholics. Rosenfeld (45) supported these findings. His findings suggest that the ingestion of 100 grams of ethanol by non-alcoholic subjects causes a marked decrease in urinary 5-HIAA excretion, during a five hour collection period.

Feldstein continued the work in this area (46). He administered serotonin-C14 to humans 30 minutes after the ingestion of ethanol and found that 5-HIAA-C14 excretion was significantly reduced in a dose-related manner.

Davis', et al., (47), results revealed a reduction in

5-HIAA-C14 along with an increase in 5-HTOH excretion. This leads one to believe that ethanol exerts its effects in man by blocking 5-HIAA formation, and shifting the metabolic pathway to a reductive one, thus forming the biogenic amine-derived alcohol (43).

Researchers have come up with two possible explanations for the altered metabolism. The first theory involves the altered NAD:NADH ratio. This ratio is altered because of metabolism of ethanol and acetaldehyde, both of which are dependent on MAO for electron transport. The lowered NAD and increased NADH levels would favor the reduction of aldehydes to the alcohol over the normal oxidation pathway to acid formation. The second theory suggests that acetaldehyde may compete for aldehyde dehydrogenase thus inhibiting the biogenic amine derived aldehydes from the oxidative pathway and shifting them to the reductive pathway, thus forming alcohols (43,46,47,48,49,50,51). There is considerable disagreement among researchers as to which explanation best describes the altered biogenic amine pathway (52). Researchers seem to agree that this alteration in pathways occurs peripherally, and not centrally. It was reported by Frankel, et al. (53) that Tyce, Flock and Owen did not observe any alteration in the metabolism of 14C-5-HT injected into the caudate nucleus of rats which had been acutely treated with ethanol. Reporting on Tytell and Myers

findings (53), they also did not observe a significant shift in metabolism to 5-HTOH in rats in the caudate nucleus, hypothalamus, and reticular formation with ethanol.

Effects of Ethanol and Acetaldehyde on Biogenic Amine Turnover and Steady-State Levels in Brain

The effect of ethanol and acetaldehyde on the steady-state levels and turnover of serotonin is a confusing issue. Gursey and Olson (54) reported that 2 grams per kilogram of ethanol given intravenously to rabbits caused a significant decrease in brain stem serotonin, up to eight hours after administration. This was not confirmed in rats by Bonnycastle, et al. (56), Pscheidt, et al. (55). Bonnycastle, et al. (56), found that one hour after ethanol administration, an increase in whole brain serotonin occurred. In an attempt to provide a possible answer to these differences by comparing the effects of ethanol on both rat and rabbit biogenic amine levels, Duritz and Truitt (56) conducted a study. Results of their study showed that ethanol at 2-4 grams per kg, intraperitoneally, had no effect on serotonin levels in either rat or rabbit brain 90 minutes after ethanol administration. The findings of Hunt and Majchrowicz (57,58) agree with those of Duritz and Truitt (56).

The results on the effect of ethanol on steady-state levels

of serotonin suggest that dosage, route of administration, duration of the study, and brain areas studied all play a role. Discrepancies in the literature will be resolved only after these factors have been standardized (43).

There have been several reports concerning the effect of acute administration of ethanol on brain 5-HT turnover. One researcher (53) reported that rat brain 5-HT turnover was slightly reduced after a single dose of ethanol (3.3 grams per kg, i.p.). Another, (53) using mice as the test animal, found that acute ethanol administration (4 grams per kg, i.p.) caused no significant change in serotonin turnover.

Palaic's results (59) include the following: ethanol (1.6 mg/kg) injected daily, produced a significant increase of 5-HT which was observed during 24 hours after the single injection of ethanol. However, following chronic treatment for 5-10 days the 5-HT content in the brain was normal. Palaic, et al. (59), found that ethanol, along with pargyline, a MAO inhibitor, potentiates the effect of pargyline on the 5-HT and 5-HIAA. Thus it appears that ethanol may actually inhibit the turnover rate of brain 5-HT in vivo. In addition, Badawy and coworkers (60) showed that acute administration of ethanol (intraperitoneally or orally) exerts a biphasic effect on the concentrations of rat brain tryptophan, 5-HT and 5-HIAA. Increases in concentration were followed by subsequent decreases. Badawy (60) suggests that this biphasic effect of ethanol on rat brain 5-HT may be due to the altered

availability of free serum tryptophan to the brain.

In summary, the effect of ethanol on the turnover of serotonin has been studied by a variety of investigators in different animals and human subjects. The data reported show that ethanol or its metabolite, acetaldehyde, exerts its effect on the normal catabolism of 5-HT. This effect results in a shift from an oxidative pathway to a reductive one where alcohol metabolites are excreted (43).

II. METHODS OF EXPERIMENTATION

Experimental Procedures and Rations

Ninety-one male weanling Sprague-Dawley rats weighing 43-58 grams were obtained and placed on a nutritionally-adequate diet for a five day acclimation period. During this period, the rats were housed according to methods described by Marsh (61).

All diets contained protein levels of approximately 30% on a dry weight basis (See Appendix A). Casein, Cheddar, Limburger and Roquefort cheese were used in this study. The composition of each diet approximated the composition of all other diets in this study, so that protein, fat and carbohydrate levels were approximately equal in each type of diet (See Appendix C). To achieve this the mixtures were made isocaloric by addition of vitamin-free dextrin. Butter fat was added to casein rations as necessary to maintain equal levels of fat. And a 4% level of standard Hegsted salt mix and 2% of a vitamin mixture were also added to each ration. The percentage composition of the vitamin and minerals has been found to be adequate for animal growth (62).

The casein had been prepared by the Sheffield Chemical Co., Norwich, New York. The mineral mixture was the Hegsted Salt Mixture from Nutritional Biochemicals Corporation, Cleveland, Ohio; and the vitamin mixture was from ICN Pharmaceuticals, Inc., Cleveland, Ohio. The rations were mixed in the kitchen

of the Animal Care Facility. To assure adequate dispersion of the ingredients, the rations were mixed in a Hobart institutional-sized Mixer for 5-10 minutes. Food was placed in glass jars, and water and/or alcohol was provided from a calibrated bottle equipped with a bent stainless steel delivery tube.

When the animals arrived, they were weighed and randomly assigned (with some adjustments to make the groups more similar) to one of eight groups. All eight groups would receive the casein diet for the first 22 days after the acclimation period. During this 22-day period, groups I-IV were force fed an alcohol solution, while groups V-VIII were given water. The alcohol drink consisted of a 5% ethanol solution to which 50 grams of sucrose/1000 ml was added. After one week, the solution was changed to a 10% solution whereupon the sucrose was removed. Alcohol consumption was monitored daily among groups I through IV by subtracting the amount wasted in the collecting bottle from the total volume of ethanol expended. After this 22-day period where groups I-IV were being "taught" to drink alcohol and groups V-VIII were not, there was a three day period where groups I-IV were to receive no alcohol in order to "deprogram" any previously learned behavior. The next 17 days consisted of the actual experimental phase of this study. During this phase, all animals (groups I-VIII) were given the choice of the alcohol

solution and/or water. Groups I and V served as control groups. These two groups received the casein diet throughout the entire experiment, while the diets were changed for groups II, III, IV, VI, VII and VIII. Groups II and VI now received a Cheddar cheese diet; groups III and VII received a Limburger cheese diet; and groups IV and VIII received a Roquefort cheese diet.

Alcohol consumption continued to be measured daily among all groups. The position of the ethanol and water bottles was rotated approximately every other day to minimize the effect of position preference (63).

Determination of Brain Serotonin

Initial Procedures

Animals were sacrificed by cervical dislocation. Brains were rapidly removed, rinsed of excess blood, labeled and frozen in dry ice, where they were kept until use.

A sensitive method for measuring 5-hydroxytryptamine developed by Curzon and Green (64) utilizing fluorometric analysis was employed in this study. All glassware was acid washed in Chromerge solution and rinsed in distilled water to prevent contaminating fluorescence.

Extraction Process

The whole brain was weighed, and then homogenized in ten

volumes of cold acidified 1-butanol. Brains were homogenized using a hand drill with a homogenizer attachment. Butanol was acidified by adding 0.85 ml concentrated HCl to 1.0 liter of 1-butanol. After homogenization of the whole brain, and centrifuging for 7 minutes at 2000 revolutions/minute in a refrigerated centrifuge at approximately 7 degrees Centigrade, the supernatant was stored in Teflon-capped test tubes in dry ice until use. 2.5 ml of the supernatant was pipetted into a 25 ml glass stoppered tube and shaken mechanically for 5 minutes with 5 ml n-heptane added to 0.4 ml 0.1 N HCl containing 0.1% L-cysteine. The contents were separated into aqueous and organic phases by centrifuging as before for 7 minutes at 2000 revolutions/minute. The aqueous phase contained the serotonin.

Serotonin Determination

To determine 5-HT, 0.1 ml aliquots of the aqueous phase were pipetted into fluorometric cuvettes, and 0.6 ml of 0.004% O-phthalaldehyde (OPT) in 10 N HCl was added. The tubes were mechanically mixed and heated in a boiling water bath for 15 minutes. 3 ml of distilled water was added to each cuvette, the tubes were wiped clean, allowed to cool and the fluorescence measured in a Turner fluorometer. Activation was at 360 nm (Turner filter #7-60, 365 nm), and fluorescence was at 470 nm (Turner filter #4, 465 nm).

Standards were prepared as 60 µg/ml or 6 mg/100 ml solutions in deionized water. The standards were diluted with a solution containing 0.1 N HCl and 0.1% cysteine, to 0.5:100, 1:100, and 2:100. 0.1 ml of the cysteine-containing solution was reacted with 0.6 ml of the 0.004% OPT in 10 N HCl solution. The standard solutions were mixed mechanically and heated in a boiling water bath for 15 minutes. Three ml of distilled water were also added to the blanks to obtain sufficient volume for reading.

Statistical Analysis

A general linear hypothesis was used to compare serotonin levels, as the dependent variable, with diet, drinking status, animal, and alcohol consumption per day as the independent variables.

A general linear hypothesis was also used to compare alcohol consumption per day, as the dependent variable, with diet, drinking status, animal, and serotonin levels as the independent variables.

Means and standard errors were calculated for alcohol consumption and serotonin levels.

III. RESULTS AND DISCUSSION

Eight groups of male weanling rats were given a nutritionally adequate casein diet. During this time, half of the animals were force fed an alcohol solution, while the other half received water. Upon completion of this phase, each group was given one of four diets: casein; Cheddar cheese; Limburger cheese; or Roquefort cheese diet; and all groups were allowed the choice of alcohol and/or water. After completion of this experimental phase, all animals were sacrificed, and a fluorometric analysis used to determine brain serotonin levels.

A general linear hypothesis was used to compare serotonin levels in all animals participating in this study. A general linear hypothesis is a statistical method whereby one may study a dependent variable in terms of one or more independent variables. The significance of these values would be examined in relation to the type of diet the animal received during the study, with drinking status (whether or not the animal had exposure to alcohol prior to the experimental phase), animal, and alcohol consumption per day as additional independent variables. The means and standard errors for the serotonin levels for each group are given in Table I,

TABLE I

MEANS AND STANDARD ERRORS FOR SEROTONIN LEVELS
(ug/g of Brain Tissue)

	Casein diet N*=12	Cheddar diet N=11	Limburger diet N=10	Roquefort diet N=9
Groups I-IV				
Mean	0.7314	1.0926	1.1538	1.2008
Std.Error	0.211	0.329	0.365	0.400
Groups V-VIII				
Mean	0.9197	1.2340	1.1310	1.4135
Std. Error	0.266	0.372	0.358	0.471

*N = Number of Animals

TABLE Ia

ANALYSIS OF VARIANCE TABLE FOR SEROTONIN LEVELS

Source	Sum of Squares	Degrees of Freedom	Mean Square	F
Drinking Status	0.3924	1	0.3924	1.47**
Diet	2.0843	3	0.6948	2.60*
Animal	2.7964	10	0.2796	1.04**
Alcohol/Day	0.1148	1	0.1148	0.43**

*p = .08; 2.76 is the cut off point for 5% level of significance - we obtained 2.60

** p greater than 0.10

with the results of the statistical analysis summarized in Table Ia.

The alcohol consumption of each animal could be examined in a similar way, using a general linear hypothesis to compare alcohol consumption in relation to type of diet being consumed. Animal and drinking status were used as additional independent variables. For the means and standard error for the alcohol consumption for each group, please refer to Table II, with the results of the statistical analysis summarized in Table IIa.

Upon referring to Table Ia, one will note the following results. The most important variable in terms of accounting for variability in serotonin levels was diet ($p=.08$). The variability due to drinking status, animal variability and alcohol consumption were not significant (p greater than .10).

Neither animal difference nor difference in drinking status were significant, however, it is of interest to note (See Table Ia) that drinking status accounts for a greater amount of variability in serotonin levels than animal difference. Referring to Table I, it is of interest to note that except for Limburger cheese where the mean values are essentially identical, the animals not having exposure to alcohol prior to the experimental phase had larger mean serotonin levels than those animals having prior exposure

TABLE II

MEANS AND STANDARD ERRORS FOR ALCOHOL CONSUMPTION
(ml)

	Casein diet N=12	Cheddar diet N=11	Limburger diet N=10	Roquefort diet N=9
Groups I-IV				
Mean	3.9	8.8	7.8	12.3
Std. Error	1.1	2.6	2.5	4.1
Groups V-VIII				
Mean	4.6	5.1	2.4	3.9
Std. Error	1.3	1.5	.8	1.3

N = Number of Animals

TABLE IIa

ANALYSIS OF VARIANCE FOR DRINKING STATUS

Source	Sum of Squares	Degrees of Freedom	Mean Square	F
Drinking Status	351.0548	1	351.0547	20.24*
Diet	163.3520	3	54.4507	3.14**
Animal	254.7634	10	25.4763	1.47

* p less than 0.0005

** cut off point for 5% level of significance = 3.15

to alcohol. None of these differences, however, were statistically significant. This suggests that the presence of cheese in the diet may not be the only variable accounting for the difference in serotonin levels, and probably accounts for the non-significance of the diet variable in the multivariate analysis. One may postulate that the level of significance for serotonin levels in relation to diet would have reached the .05 level had there not been the confounding variable, alcohol consumption, and had there been a larger sample size. Note there was a definite trend in this direction - for an F ratio critical at the .05 level, 2.76 was needed for significance, and the F ratio obtained was 2.6.

In summary, findings from this study show that serotonin levels may be the result of an interaction between type of diet and alcohol status, i.e., whether the animal drank alcohol throughout the entire course of the experiment, or merely had access to it during the experimental phase.

In an attempt to explain why the difference in serotonin levels for those having been taught to drink alcohol, and those not, we will refer back to the work of Palaic, et al. (59). Palaic found that the 5-HT level of rat brain was significantly increased by acute ethanol treatment (1.6 mg/kg, i.p.), while it remained normal during chronic treatment for 5-10 days. In his studies, Palaic also found that ethanol potentiates both the effects of the drugs

pargyline and pCPA on 5-HT and 5-HIAA levels in the brain, indicating an inhibitory effect upon the turnover rate of brain 5-HT. If this is true, and assuming that the tyramine from the cheese, because of its competition for MAO, acts like the MAO inhibitor, pargyline, it could be a possible explanation for the increased 5-HT levels in the animals who drank only small amounts of ethanol during the experimental phase. That is, the higher 5-HT levels observed in these animals may reflect a slow rate of 5-HT degradation through the alternate metabolic route (5-HT to 5-HTOH instead of 5-HT to 5-HIAA), giving rise to the accumulation of endogenous 5-HT.

In conjunction with these observations, Badawy and Evans (60) have noted that acute administration of ethanol exerts a biphasic effect on the concentrations of rat brain tryptophan, 5-HT, and 5-HIAA. Results show that in addition to the initial increases in the above concentrations, ethanol causes subsequent decreases. Both aspects of this biphasic effect are associated with an altered availability of circulating free tryptophan. The results of this study may reflect a combination of effects exerted by altered synthesis and turnover rates of 5-HT.

The means and standard errors for alcohol consumption for each group are given in Table II, with the results of the statistical analysis summarized in Table IIa.

One will note the following results. The most important variable in terms of accounting for variability in alcohol consumption was drinking status, i.e., whether or not the animal had exposure to alcohol prior to the experimental phase ($p < 0.0005$). In other words, the amount of alcohol drunk during the experimental phase was very dependent on whether or not the animal had been "taught" to drink prior to this phase. Those having been "taught" drank, on the average, about twice as much as those not taught, e.g. means = 7.93 and 4.01, respectively.

With respect to those groups (I-IV) having prior exposure to alcohol, the alcohol consumed among those on the cheese diets was significantly larger than those on the casein diet ($p=0.05$). While those groups (V-VIII) having no prior exposure to alcohol, consumption of alcohol did not significantly increase in those animals on a cheese diet. These results may suggest that diet is not highly important in acute alcohol consumption, but in terms of more chronic alcohol consumption, diet may be a factor in terms of the ability to control drinking.

Further Research Suggested by this Project

Suggestions for further refinement of the research carried out in this project might include alterations in the experi-

mental design. One such alteration would be the inclusion of a group of animals who would remain naive to alcohol. Had there been such a group in the present study, more substantial data might have been obtained. Another alteration in the design, to be recommended, would be the measurement of alcohol consumption once a week, instead of daily, as was done in the present study. This would tend to lessen the effects of measurement error. One more alteration to consider might be having only one person extract the brains from the animals. In the present study, the removal of the brains was performed by two persons. This may have led to variability in the amount of brain and brain stem extracted.

Further research might also explore the relation in human beings of diet to alcohol consumption. The results of this study suggest that in terms of chronic alcohol consumption, diet may be a factor in the ability to control drinking. If this were also true in human beings, its implications for the recovery of alcohol abuse might be far-reaching.

IV. SUMMARY

Findings suggest that diet appears to play a role in predicting brain serotonin levels ($p=.08$), and may have been a stronger predictor had the variable, alcohol consumption, not been introduced into the design.

Results also suggest that serotonin levels are the result of an interaction between type of diet and total alcohol consumption. This is supported by the fact that there were significant differences (p less than .05) in serotonin levels among those animals having prior exposure to alcohol and those not having prior exposure to alcohol for the casein, Cheddar and Roquefort cheese diets.

Results further show mean serotonin levels, in general, are significantly lower (p less than .05) among those animals that drank alcohol throughout the entire course of the experiment versus those animals that had not drunk alcohol prior to the experimental phase of this study. These results coincide with previous research (66) showing that ethanol is able to lower brain serotonin levels.

Finally, drinking status appeared to be the strongest predictor of subsequent alcohol consumption. An interesting finding from this study was that those animals having prior exposure to alcohol (groups I-IV), consumed significantly larger amounts of alcohol when on the cheese diets, than

among those on the casein diet (p=.05). While those groups (V-VIII) having no prior exposure to alcohol, did not consume significantly larger amounts of alcohol when on one of the cheese diets. This may suggest that diet is not highly important in acute alcohol consumption, but in terms of more chronic alcohol consumption, diet may be a factor in terms of the ability to control drinking.

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APPENDIX A.

PERCENT COMPOSITION OF RATIONS ON A DRY WEIGHT BASIS

Protein Source	Protein	Fat	Dextrin	Min.	Vit.
Casein	30%	40%	24%	4%	2%
Cheddar	30	40	24	4	2
Limburger	30	40	24	4	2
Roquefort	30	43	21	4	2

APPENDIX B.

INGREDIENTS OF EXPERIMENTAL DIETS

Cheddar Cheese Diet: (Groups II & VI - 23 animals)	22 kg Cheddar 4.4 kg Dextrin 368 g Vitamin mix 736 g Mineral mix
Limburger Cheese Diet: (Groups III & VII - 23 animals)	27.2 kg Limburger 4.3 g Dextrin 368 g Vitamin mix 736 g Mineral mix
Roquefort Cheese Diet: (Groups IV & VIII - 22 animals)	24.2 kg Roquefort 3.76kg Dextrin 352 g Vitamin mix 704 g Mineral mix
Casein Diet: (Groups I & V - 22 animals for 16 days & 91 animals during initial 24 days)	40.4 kg Casein 60.8 kg Butterfat 32.28kg Dextrin 2.54kg Vitamin mix 5.08kg Mineral mix

Cheese Diets calculated for a 16-day period.

APPENDIX C.

PREPARATION OF DIET

	Experimental Groups							
Diet Component	I	II	III	IV	V	VI	VII	VIII
Casein	x				x			
Cheddar		x				x		
Limburger			x				x	
Roquefort				x				x
Dextrin	x	x	x	x	x	x	x	x
Butterfat	x				x			
Salt Mix	x	x	x	x	x	x	x	x
Vitamin Mix	x	x	x	x	x	x	x	x
Alcohol for 1st 22 days	x	x	x	x				

APPENDIX D.

STANDARD CURVE